PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/53, 9/02, C12Q 1/26, C12N 1/21, 1/19 // (C12N 1/21, C12R 1:19) (C12N 1/19, C12R 1:865)

A1

(11) International Publication Number: WO 96/22376

(43) International Publication Date: 25 July 1996 (25.07.96)

(21) International Application Number: PCT/GB96/00099

(22) International Filing Date: 19 January 1996 (19.01.96)

(30) Priority Data:

9501172.2	20 January 1995 (20.01.95)	GB
9508301.0	24 April 1995 (24.04.95)	GB

(71) Applicant (*for all designated States except US*): THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, DRA Farnborough, Hampshire GU14 6TD (GB).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): SQUIRRELL, David, James [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). LOWE, Christopher, Robin [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). WHITE, Peter, John [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). MURRAY, James, Augustus, Henry [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB).

(74) Agents: BOWDERY, Anthony, Oliver et al.; Defence Evaluation & Research Agency, Intellectual Property Dept., R69 Building, DRA Farnborough, Hampshire GU14 6TD (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MUTANT LUCIFERASES

(57) Abstract

Proteins are provided having luciferase activity with lower K_m than wild-type luciferases by altering the amino acid residue at position 270 of the wild-type to an amino acid other than glutamate. Greater heat stability than wild-type luciferases while retaining the lower K_m is provided by also replacing the glutamate equivalent to that at position 354 of *Photinus pyralis* luciferase or 356 of *Luciola* luciferases with an alternative amino acid, particularly lysine and/or the amino acid residue at 215 of *Photinus pyralis* and 217 of the *Luciola* species with a hydrophobic amino acid. DNA, vectors and cells that encode for and express the proteins are also provided as are test kits and reagents for carrying out luminescence assays using the proteins of the invention.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

MUTANT LUCIFERASES

The present invention relates to novel proteins having luciferase activity and to DNA and vectors encoding for their expression. Particularly the present invention provides luciferases having lower K_m for the substrate ATP than existing native and recombinant luciferases of wild and altered wild type.

Firefly luciferase catalyses the oxidation of luciferin in the presence of ATP, Mg^{2+} and molecular oxygen with the resultant production of light. This reaction has a quantum yield of about 0.88 (see DeLuca & McElroy (1978) and Seliger & McElroy (1960)) and this light emitting property has led to its use in luminometric assays where ATP levels are being measured.

Luciferase is obtainable directly from bodies of fireflies or by expression from microorganisms including recombinant DNA constructs encoding for the enzyme. Significant species from which the enzyme may be obtained, or DNA encoding for it derived, are the Japanese GENJI and HEIKE fireflies Luciola cruciata and Luciola lateralis, the East European Firefly Luciola mingrelica, the North American firefly (Photinus pyralis) and the glow-worm and the European glow-worm Lampyris noctiluca.

The heat stability of wild and recombinant type luciferases is such that they lose activity quite rapidly when exposed to temperatures in excess of about 30°C, particularly over 35°C, and this renders the enzyme deficient when used at high ambient temperatures. It is known that Japanese firefly luciferase can be heat stabilised by mutating it at its position 217 to replace a threonine residue by an isoleucine residue (Kajiyama and Nakano (1993) Biochemistry 32 page 13795 to 13799); pH stability and specific activity also being increased.

Copending patent application GB 9405750.2 discloses an amino acid substitution that is capable of increasing the thermostability of *inter alia*, Photinus pyralis which can be used with the change at 217 to provide luciferase that is relatively heat stable at 50°C or more.

The present invention relates to a further enhancement of the properties of luciferase enzymes, making them suitable for use in assays based upon the detection of adenosine triphosphate at relatively low levels. This enhancement is provided by changing the amino acid at the position corresponding to position 270 in the Photinus pyralis luciferase amino acid sequence whereby the Michaelis-Menten constant (K_m) of the enzyme is decreased as compared to a corresponding luciferase having wild-type sequence. This corresponds to amino acid 272 in Luciola mingrelica, Luciola cruciata and Luciola lateralis. It also corresponds to amino acid 270 in Lampris Noctiluca.

The present enhancement further provides luciferases that are characterised by the ability oxidise D-luciferin with light emission of a different wavelength to that of wild-type luciferase, thus allowing them to be used as specific labels in binding assays wherein the wavelength of light emitted is characteristic of a particular labelled material being present, or allows DNA encoding for the luciferases to be used as a reporter DNA for genetically engineered cells and cells derived therefrom.

Thus in the first aspect of the present invention there is provided a protein having luciferase activity and having over 60% homology of amino acid sequence with that of Photinus pyralis, Luciola mingrelica, Luciola cruciata or Luciola lateralis characterised in that the amino acid residue corresponding to residue 270 of Photinus pyralis luciferase and residue 272 of Luciola mingrelica, Luciola cruciata and Luciola lateralis luciferase is an amino acid other than glutamate. Preferably is characterised in that it comprises a conserved amino acid sequence F(1)XE(2)FL wherein (1) is D or E, (2) is T or L and X is the amino acid other than glutamate; F, E, L, D and T each relating to the corresponding amino acid as provided for by the single letter amino acid code.

The preferred amino acid X so far determined is lysine, or an analogue or modification thereof. Other preferred amino acids include arginine, glutamine and alanine.

In still more preferred forms of the present invention the protein of the invention also has the amino acid at the position corresponding to amino acid 217 of the Luciola firefly luciferases or 215 of Photinus pyralis changed to a hydrophobic amino acid, preferably to isoleucine, leucine or valine or analogue or these and/or has the amino acid at the position corresponding to amino acid 356 of the Luciola firefly luciferase or 354 of Photinus pyralis changed to an amino acid other than glutamate, particularly to lysine, arginine, leucine, isoleucine or histidine or analogues or modifications of these.

In a second aspect of the invention there is provided DNA encoding for the protein of the invention and in a third aspect there is provided a vector, particularly a plasmid, comprising a *luc* gene (the gene encoding for luciferase) in such a form as to be capable of expressing the protein of the invention. Such forms are those where the vector includes DNA sequences capable of controlling the expression of the protein of the invention such that when incorporated into a microorganism host cell the protein may readily be expressed as required, if necessary by addition of suitable inducers.

The *luc* genes for Photinus pyralis, Luciola mingrelica, Luciola cruciata and Luciola lateralis are all known and isolatable by standard molecular biology techniques. This is also the case for Lampris noctiluca. Photinus pyralis *luc* gene is commercially available from Promega as the plasmid pGEM-luc. Thus convenient methods and sources for deriving starting material for production of DNA of the invention are (i) use of naturally occurring firefly genomic DNA and amplifying the *luc* gene from it using eg, PCR, (ii) pGEM and (iii) pGLf37 plasmid of Kajiyama and Nakano. Further genes encoding for proteins having luciferase activity, ie the activity of oxidising luciferin with the emission of light, will also be suitable sources for starting material for obtaining a DNA, and ultimately through gene expression, a protein of the invention.

Suitable vectors for use in manipulating wild type or other *luc* gene DNA in order to produce the DNA of the invention will be any vector in which the DNA can be contained within while alteration of the naturally occurring glutamate to an alternative amino acid is carried out. For chemically induced mutagenesis, eg using agents such as hydroxylamine, this is not particularly critical and many suitable vectors will occur to those skilled in the art that will allow easy manipulation of the gene before and after the mutagenic process. It may be preferred to specifically mutate the *luc* gene at the glutamate and thus a site directed mutagenesis operation will be required. Such operations may be most easily carried out in vectors and these will be well known to those skilled in the art.

For expression of *luc* genes of wild and known type, and those of the present invention suitable vectors include pKK223-3, pDR540 (available from Boehringer Mannheim) and pT7-7; the first two having the tac promoter under control of the lactose repressor allowing expression to be induced by presence of isopropyl-thiogalactoside (IPTG). pT7-7 allows control by the T7-RNA polymerase promoter and thus provides the basis for a very high level of gene expression in E. coli cells containing T7 RNA polymerase. Of these vectors expression is found to be highest when the *luc* genes are inserted into the pT7-7 vector.

Expression of luciferase from a *luc* gene inserted into pKK223-3 and pDR540 results in the expression of wild-type N-terminal sequence luciferase whilst expression from a *luc* gene inserted into pT7-7 results in synthesis of a fusion protein with extra N-terminal amino acids A-R-I-Q. The ribosome binding site and start codon of the *luc* gene in each of the respective vectors with the *luc* gene present (named constructs pPW204, pPW116 and pPW304) are shown in Table I of the Examples. pPW601a referred to below is derived by removing the unique Xho I site pPW116.

A third aspect of the present invention provides cells capable of expressing the proteins of the invention; methods for producing such proteins using these cells and test kits and reagents comprising the proteins of the invention. Also provided are assay methods wherein ATP is measured using luciferin/luciferase reagents, as is well known in the art, characterised in that the luciferase is a protein of the invention. Luciferase preparations of the invention are relatively low in K_m with respect to the corresponding wild type and recombinant luciferases, and preferred double and triple change luciferases (ie 215; 270; 354 changed Photinus or 217; 272; 356 changed Luciola, or 215; 270; 354 changed L. noctiluca also have the property of relative thermostability at 50-70°C, particularly 37-60°C, and especially 40-50°C. Thus the present invention has been established as not preventing the thermostability enhancements of other contemporaneous and previous work by the present inventors and others from being used.

Any cell capable of expressing heterologous protein using DNA sequences in its DNA, or in vectors such as plasmids contained in the cell, may be used to express the proteins of the invention. Typical of such cells will be yeast and bacterial cells such as Saccharomyces cerevisiae and Escherichia coli cells, but many other host organisms suitable for the purpose of protein expression will occur to those skilled in the art.

The protein may be expressed as a protein of similar structure to native and known recombinant luciferases, or may be expressed as a fusion or conjugate of such proteins with other amino acids, peptides, proteins or other chemical entities, eg the A-R-I-Q sequence above.

It will be realised by those skilled in the art that certain hosts may have particular codon preferences, eg bacteria in some cases use different codons to yeast, and thus the DNA incorporated into such a host may advantageously be altered to provide a degenerate codon for a given amino acid that will give more favourable expression in that host. Such degenerate DNAs are of course included in the scope of the DNA of the invention.

E. coli BL21 (DE3) is one suitable host and has the T7 RNA polymerase integrated stably into its chromosome under control of the inducible lacUV5 promoter and is thus compatible with pT7-7 derived constructs.

E. coli B strains like BL21 lack the *lon* protease and the *ompT* outer membrane protease. These deficiencies can help to stabilise the expression and accumulation of foreign proteins in E. coli. Assays of crude extracts of E. coli BL21 (DE3) containing each of the three expression constructs described above indicated that the highest levels of expression of luciferase were obtained from cells containing the construct pPW304 (see Table 2). Other suitable cell lines, such as that of the E. coli JM109 cells used in the Examples below will occur to those skilled in the art.

The proteins, DNA, vectors and cells of the invention will now be described by way of illustration only by reference to the following non-limiting Examples. Figures, Tables and Sequence listing. Further proteins, conjugates of proteins, DNA, vectors and cells, and assays and test kits incorporating any of the above will occur to those skilled in the art in the light of these.

FIGURES

Figure 1: shows a restriction map of plasmid pPW204 derived from pKK223-3 by insertion of a *luc* gene as described in the Examples below.

Figure 2: shows a restriction map of plasmid pPW116 derived from pDR540 by insertion of a *luc* gene as described in the Examples below.

Figure 3: shows a restriction map of plasmid pPW304 derived from pT7-7 by insertion of a *luc* gene as described in the Examples below.

Figure 4: shows a restriction map of plasmid pPW601a derived from pDR540 and BamH1/Sst1 fragment from pGEM-*luc* with the Xho site removed.

Figure 5: shows a graph of heat inactivation of recombinant wild-type Photinus luciferases (Sigma), K_m changed luciferase of the invention, the thermostable 354 lysine mutant provided by copending GB 9405750.2 and K_m/354 lysine double mutant of the present invention incubated at a given temperature for 16 minutes as described in the Examples below.

Figure 6: shows a restriction map of pT7-7 after Tabor.

SEQUENCE LISTING:

The sequence listing provided at the end of this specification describes DNA and amino acid sequences as follows:

SEQ ID No 1: shows the DNA sequence of a DNA encoding for luciferase of the invention wherein the Photinus pyralis wild-type codon at 811 to 813 is mutated; for lysine only the base at 811 is mutated to an A. It also shows the position for introducing thermostability at 1063-65.

SEQ ID No 2: shows the amino acid sequence of a protein of the invention wherein the Photinus pyralis wild-type amino acid 270 glutamate has been changed to a residue Xaa other than glutamate.

SEQ ID No 3: shows the sequence of the oligonucleotide used for the SDM mutation of pPW601a to give a lysine instead of glutamate at position 270.

SEQ ID No 4: shows the amino acid sequence of a protein of the invention wherein the Photinus pyralis wild-type amino acid 270 glutamate has been changed to lysine and the 354 amino acid changed to lysine.

SEQ ID No 5: shows the sequence of the oligonucleotide used for the SDM mutation of pPW601a to give a lysine instead of glutamate at position 354.

EXAMPLES

Example 1: Production of plasmids containing DNA of the invention

Plasmids pKK223-3 and pDR540 were obtained form Boehringer Mannheim; pDR540 is also available from Pharmacia Biotech St Albans UK. Phagemid pBluescript II SK(+) was obtained from Stratagene La Jolla, USA. E. coli strain BL21 (DE3) was used for the expression of luciferase from pT7-7 derived plasmids and E. coli strain JM109 (4) was used in all cloning experiments and for the expression of luciferase from pDR540 derived plasmids.

Plasmid pT7-7 (see Current protocols in Molecular Biology Vol II Section 16.2.1) was obtained from Stan Tabor, Dept of Biol Chem, Harvard Medical School, Boston, Mass 02115 and (as shown in Figure 6) contains T7 RNA polymerase promoter ø10 and the translation start site for the T7 gene 10 protein (T7 bp 22857 to 22972) inserted between the PvuII and ClaI sites of pT7-5. Unique restriction sites for creation of fusion proteins (after filling in 5' ends) are Frame 0: EcoR1; Frame 1: NdeI, SmaI, ClaI; Frame 2: BamHI, SalI, HindIII. SacI site of the original polylinker is removed by deletion and an additional XbaI site is provided upstream of the start codon.

As stated in the preamble to the Figures, pPW204 was derived from pKK223-3; pPW116 was derived from pDR540; pPW304 derived from pT7-7; each by insertion of a *luc* gene derived from Promega pGEM-*luc* using standard restriction endonuclease and ligation techniques while pPW601 was created by cloning the *luc* gene and BamH1/Sst1 fragment from pGEM-*luc* into pDR540 and pPW601a as derived by removing the unique Xho I site in the polylinker of the plasmid. pPW601a contains a unique recognition site for Ava I which simplifies the SDM procedure for luciferase amino acid 354 changes.

For production of pPW304, pT7-7 is digested with EcoRI, the ends filled using Klenow fragment, the product digested with SalI and the DNA gel purified; pGEM-*luc* is digested with Bam HI, the overhangs produced digested with MBN, the product digested with SalI and the 1.Kb fragment produced purified and ligated to the purified pT7-7 DNA.

Transformation of plasmids into BMH 71-18 mut S cells was carried out using a Bio-Rad Gene Pulser version 2-89. For production of pPW601 clones harvested cells and purified mixed plasmid pool containing mutated and parental plasmids were provided and secondary restriction digest with AvaI was carried out before transformation into E. coli JM109 cells. These cells were plated on selective media (LB agar + 50 μg/ml ampicillin) and clones screened by purifying their plasmid DNA and analysing for the desired change. Plasmid DNA was purified using alkaline lysis (Birnboim & Doly (1979) Nucleic Acids Research 7, p1513).

Relative levels of expression of luciferase from each of the constructs pPW204, pPW116 and pPW304 are 0.1: 0.5: 1.0 from E. coli BL21 (DE3). Cells were grown in LB at 37°C to an OD 600 of 0.3 then induced with IPTG and growth allowed to continue for 4 hours after which crude extract was prepared and luciferase activity measured.

TABLE 1: Ribosome binding sites (underlined) and start codons in the expression constructs used in Example 1.

pPW304	AAGGAGATATACAT ATG* CGT AGA ATT CAA ATG
pPW116	AGGAAACAGGATCCA ATG*
pPW204	AGGAAACAGCAA ATG*

Partial purification of luciferases was carried out on E. coli JM109 cells harvested in the early stationary phase then resuspended in 50mM Tris HCl pH 8.0 containing 50mM KCl, 0.5mM dithiothreitol and 1 mM EDTA (Buffer A). Cells were broken up by disruption in a MSE soniprep (amplitude 14μ) and the lysate centrifuged at 30000 x g for 1 hour. The supernatant of the crude extract was then subjected to fractionation with ammonium sulphate and the fraction precipitated between 35% and 55% saturation contained luciferase activity and was dissolved in Buffer A and dialysed overnight against 500ml of 50mM Tris-HCl buffer pH8.0 containing 0.4mM DTT (Buffer B).

Full purification of luciferases was carried out by applying the precipitated and dialysed enzyme to a Mono Q (HR10/10) anion exchange column and eluting that with a linear gradient of 0-200mM NaCl in Buffer B (flow-rate 4ml/minute; 2ml fractions). Peak fractions containing luciferase activity were made to 50% glycerol (v/v) and stored at -20°C.

Firefly luciferase (prepared from a crystalline suspension, Cat No L9009) and coenzyme A and ATP were obtained from Sigma Chemical Co. Beetle luciferin potassium salt was obtained from Promega Corporation, Madison Wisconsin, USA. Cell extracts were prepared as described in the Promega technical bulletin No 101. Aliquots of E. coli cultures were lysed in cell culture lysis reagent (25mM Tris-phosphate, pH7.8, 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X-100, 2.5 mg/ml BSA, 1.25 mg/ml lysozyme) for 10 minutes at room temperature, centrifuged at 16000g for 2 minutes and then stored on ice prior to assay.

Luciferase activity of cell lines was assayed by monitoring bioluminescence emitted by colonies by transferring these to nylon filters (Hybond N, Amersham) and then soaking the filters with 0.5mM luciferin in 100mM sodium citrate buffer pH5.0 (Wood & DeLuca, (1987) Anal Biochem 161 p501-507) at room temperature. Luciferase assays in vitro were performed at 21°C using 100µl of assay buffer (20mM Tricine pH7.8 containing 1mM $MgSO_4$, 0.1mM EDTA, 33.3mM DTT, 0.27mM conenzyme A, 0.47mM D-luciferin, 0.53mM ATP and 1 to 5µl of sample). The final pH of the assay cocktail was 7.8 and light measurements were made with a BioOrbit 1250 luminometer or in microtitre plates using a labsystems luminoskan RS plate luminometer.

Protein was determined by the method of Bradford (1976) Anal. Biochem. 72 p248-254 using BSA as standard. For production of non-specific chemical mutations of DNA, plasmids containing *luc* genes were treated according to the method of Kironde et al (1989) Biochem. J. 259, p421-426 using 0.8M hydroxylamine, 1mM EDTA in 0.1mM sodium phosphate pH6.0 for 2 hours at 65°C.

The K_m mutant was initially generated by hydroxylamine induced mutagenesis of the *luc* gene within pPW304 to provide plasmid 304 G1 bearing a single base change in the DNA sequence at 811 of SEQ ID No 1 resulting in an amino acid glutamate change to lysine at position 270. A 1.1kb DNA fragment (BstE II/Stu I) was cloned from pPW304 and used to replace the corresponding fragment in pP601a to form pPW601G1, thus providing a *luc* gene encoding for luciferase without the four extra amino acids encoded by pPW304 (M not included from M-A-R-I-Q).

This mutagenised plasmid was desalted on a G60 DNA grade Nick column (Pharmacia) followed by transformation into E. coli BL21 (DE3). Luciferase expressed from this showed an identical low K_m phenotype to that of the original mutant.

Double stranded DNA sequencing was performed by the dideoxy chain termination method of Sanger et al (1977) Proc. Nat. Acad. Sci. (USA) 74, 5463-5467 using [alpha-^{32}P]dATP and electrophoresis in 8M urea (6% wt/vol) polyacrylamide gels. Automatic sequencing was also undertaken using a DNA model 373A automated sequencer (Applied Biosystems).

Assay for determining the K_m value of this luciferase with respect to ATP was carried out at 21°C with 100µl of assay buffer (20mM tricine pH7.8 containing 1.0mM $MgSO_4$. 0.1mM EDTA, 33mM dithiothrietol, 270µM coenzyme A, 470µM D-luciferin and 6.25 to 400µM ATP) using a luminometer to measure cpm.

The K_m value for 601a-recombinant wild-type was determined to be 66.1µM (s.e. 4.1); for 601aK (thermostable mutant 354 lysine) was 61.3 (s.e. 4.7) and for 601aG1 (270 lysine K_m change) was 28.7 (s.e. 0.9) thus illustrating that the 270 change more than halves the ATP concentration for which the enzyme is optimised.

The effect of the 270 change on the thermostability of luciferase is negative, with $t_{1/2}$ activity being reached after only 2 minutes as compared to wild-type at 7 minutes, both at 37°C; however at 30°C the specific activity of 270 is greater than wild-type.

Example 2: Preparation of 'double mutant' 270K: 354K Photinus pyralis luciferase

In order to offset the reduced thermostability of the 270 change luciferase, a double change luciferase was provided by using site directed mutagenesis to engineer the lysine change at 354 into the 270-lysine luciferases encoding DNA and plasmid described in Example 1. This involved mutation using specifically designed oligonucleotides to convert pPW601aG1 to pPW601a to G1K.

The oligonucleotide used to generate the 354 lysine change by SDM was CATCCCCCTTGGGTGTAATCAG (SEQ ID No 5) with the underlined T being the mismatch.

The site directed mutagenesis required to convert the glutamate 354 of pPW601aE270K, and where required for direct synthesis of 270 mutant from pPW601a, to desired amino acids is carried out using the following protocol with oligonucleotides designed as required.

Site Directed Mutagenesis Protocol: Plasmid selected is denatured and annealed with selection and mutagenic oligonucleotides for the desired change. The mutant DNA strand is synthesised and ligated and the whole primary restriction digested with a restriction endonuclease. Oligonucleotide primers for sequencing and SDM were synthesised using an Applied Biosystems model 380A DNA synthesiser. DNA oligonucleotide primers were designed to destroy either a unique Ava I site within the *luc* gene or the unique ScaI site within the gene for β-lactamase; the presence of these sites being used to select against plasmids that had not undergone mutagenesis. Precise protocols were as described in the Transformer^{RTM} Site -Directed Mutagenesis Kit (Version 2.0) sold by Clontech Laboratories Inc (US) catalog No K1600-1.

The restriction map for pPW601 derived from pDR540 and cloned *luc* gene is shown as Figure 4. Site directed mutagenesis was carried out as described above and in the Clontech instructions such as to convert the wild-type Photinus *luc* gene inserted therein into a sequence as shown in SEQ ID No 1 with expressed protein of amino acid sequence modified at position 270 as shown as Xaa in SEQ ID No 2 to Lysine.

K_m studies were carried out as described in Example 1 while heat inactivation studies were carried out using crude extracts at 37°C in lysis buffer (25mM Tris phosphate pH7.8, 2mM DTT, 2mM EDTA, 10% glycerol and 1% Triton X-100) at various time points aliquots of enzyme were removed and assayed as described above (with 530μM ATP). The remaining activity was plotted against time.

The K_m value for 601aG1K, the double change of this example, was found to be 25.2μM (s.e. 1.5) being again less than half that of the corresponding 354 lysine mutant and the wild-type luciferase.

The $t_{1/2}$ value, the time after which the activity of the luciferase is reduced on continuous heating to 50% of its initial value, was found to be as follows:

601a	(recombinant wild-type)	$t_{1/2}$ reached after 7.0 minutes
601aG1	(270 K_m change)	$t_{1/2}$ reached after 1.75 minutes
601aK	(354 thermostable change)	$t_{1/2}$ reached after >35 minutes
601aG1K	(270 + 354 change)	$t_{1/2}$ reached after 10.5 minutes

The above data is included below (plus other data) along with Km values.

	Km ATP	$t_{1/2}$ 37°C (min)
recombinant wild type	66.1	7.0
E270K	28.7	1.75
E354K	61.3	>35
E270K + E354K	25.2	10.5
E270R	32.0	1.75
E270Q	44.0	1.75
E270A	37.0	1.75

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SECRETARY OF STATE FOR DEFENCE. .

(ii) TITLE OF INVENTION: luciferases

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: THE SECRETARY OF STATE FOR DEFENCE
(B) STREET: WHITEHALL
(C) CITY: LONDON
(D) STATE: LONDON
(E) COUNTRY: UNITED KINGDOM (GB)
(F) ZIP: SW1A 2HB

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1722 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 4..1653

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(811..813, "")

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAATGGAAG ACGCCAAAAA CATAAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT 60

GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA 120

ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG 180

TCCGTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC 240

GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA 300

GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC 360

ATTTCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG 420

CAAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG 480

GGATTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC 540

GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT 600

GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC 660

TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT 720

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA 780

TTTCGAGTCG TCTTAATGTA TAGATTTGAA NNNGAGCTGT TTTTACGATC CCTTCAGGAT 840

TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT 900

CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT 960

TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATT TTCCAGGGAT ACGACAAGGA 1020

TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCNNNGGGGA TGATAAACCG 1080

GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GTAGCGAAGG TTGTGGATCT GGATACCGGG 1140

AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC 1200

GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT 1260

TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG 1320

TCTTTAATTA AATACAAAGG ATATCAGGTC GCCCCCGCTG AATTGGAATC GATATTGTTA 1380

CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA 1440

CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG 1500

GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG 1560

GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC 1620

ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC 1680

GAAATTCTTA GCTATTGTAA TCCTCCCAGG CCTCGAGGTC GA 1722

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 270

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1               5                  10                  15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
            20                  25                  30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
        35                  40                  45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
    50                  55                  60
```

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Xaa Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(10, "")

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATAGATTT GAAAAAGAGC TGTTTTTACG 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Photinus pyralis

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 354

(ix) FEATURE:

(A) NAME/KEY: Modified-site

(B) LOCATION: 270

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1               5                  10                  15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
            20                  25                  30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
        35                  40                  45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
    50                  55                  60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65                  70                  75                  80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
                85                  90                  95
```

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Leu Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His His Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Lys Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Lys Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: misc_difference
(B) LOCATION: replace(10, "")

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATCCCCCTT GGGTGTAATC AG 22

CLAIMS

1. A protein having luciferase activity and having over 60% homology of amino acid sequence to luciferase from Photinus pyralis, Luciola mingrelica, Luciola cruciata or Luciola lateralis characterised in that the amino acid residue corresponding to residue 270 of Photinus pyralis luciferase or residue 272 of Luciola mingrelica, Luciola cruciata and Luciola lateralis luciferase is of an amino acid other than glutamate.

2. A protein as claimed in claim 1 characterised in that it comprises an amino acid sequence F(1)XE(2)FL wherein (1) is D or E, (2) is T or L and X is the amino acid residue other than glutamate.

3. A protein as claimed in claim 2 characterised in that it comprises an amino acid sequence TPXGDDKPGA wherein X is the amino acid residue other than glutamate.

4. A protein as claimed in claim 1, 2 or 3 characterised in that the amino acid residue X is lysine.

5. A protein as claimed in any one of claims 1 to 4 wherein the amino acid residue corresponding to that of residue 215 of Photinus pyralis luciferase or residue 217 of Luciola mingrelica, Luciola cruciata or Luciola lateralis luciferase is of a hydrophobic amino acid.

6. A protein as claimed in claim 5 wherein the residue corresponding to that of residue 215 of Photinus pyralis luciferase or residue 217 of Luciola mingrelica, Luciola cruciata or Luciola lateralis luciferase is one of isoleucine, leucine or valine or an analog of any of these.

7. A protein as claimed in any one of claims 1 to 6 wherein the amino acid residue corresponding to that of residue 354 of Photinus pyralis luciferase or residue 356 of Luciola mingrelica, Luciola cruciata or Luciola lateralis luciferase is of an amino acid other than glutamate.

8. A protein as claimed in claim 7 wherein the residue corresponding to that of residue 354 of Photinus pyralis luciferase or residue 356 of Luciola mingrelica, Luciola cruciata or Luciola

lateralis luciferase is one of lysine, arginine, leucine, isoleucine or histidine or an analog of any of these.

9. A protein comprising an amino acid sequence as described in SEQ ID No 2 wherein Xaa is lysine.

10. A DNA encoding for a protein as claimed in any one of claims 1 to 9.

11. A DNA as claimed in claim 10 comprising a nucleotide sequence as described in SEQ ID No 1 wherein the three bases N at 811 to 813 form a codon encoding for an amino acid other than glutamate.

12. A DNA as claimed in claim 11 wherein the codon encodes for lysine.

13. A vector comprising a *luc* gene encoding for a protein as claimed in any one of claims 1 to 9.

14. A vector as claimed in claim 13 obtainable by treating a vector containing a wild-type or recombinant *luc* gene by site directed mutagenesis to change the codon responsible for encoding for the glutamate at position 270 of Photalis pyralis luciferase or the glutamate at position 272 of Luciola mingrelica, Luciola cruciata or Luciola lateralis luciferase to an alternative amino acid.

15. A vector as claimed in claim 14 wherein the alternative amino acid is lysine.

16. A vector as claimed in any one of claims 13 to 15 selected from pKK223-3, pDR540 and pT7-7 into which a *luc* gene has been ligated.

17. A cell capable of expressing a protein as claimed in any one of claims 1 to 9 comprising DNA or a vector as claimed in any one of claims 10 to 16.

18. A cell as claimed in claim 17 being an E. coli or a S. cerevisiae.

19. A test kit for performance of an assay through measurement of ATP characterised in that the kit comprises a protein as claimed in any one of claims 1 to 9 contained within a luminescent reagent.

20. An assay method wherein ATP is measured using luciferin and luciferase to generate light the quantity of which is related to the amount of ATP characterised in that the luciferase is a protein as claimed in any one of claims 1 to 9.

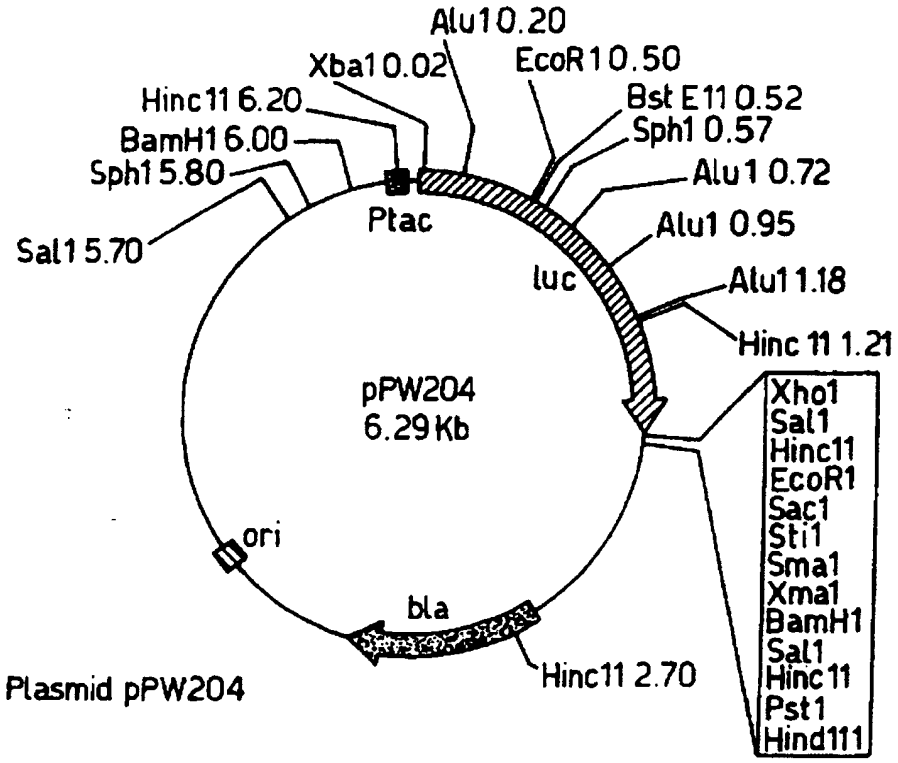
21. An assay method as claimed in claim 20 wherein the assay is carried out at a temperature of from 30°C to 70°C.

22. An assay method as claimed in claim 20 wherein the assay is carried out at a temperature of from 37°C to 60°C.

23. An assay method as claimed in claim 20 wherein the assay is carried out at a temperature of from 40°C to 50°C.

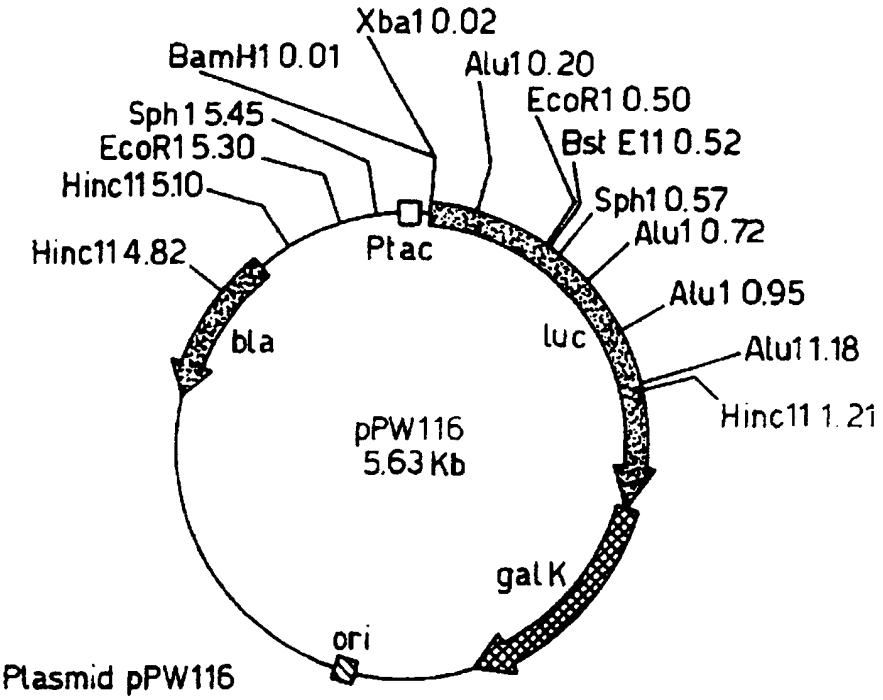
24. A protein comprising an amino acid sequence as described in SEQ ID No 2 wherein Xaa is chosen from arginine, glutamine and alanine.

Fig.1.



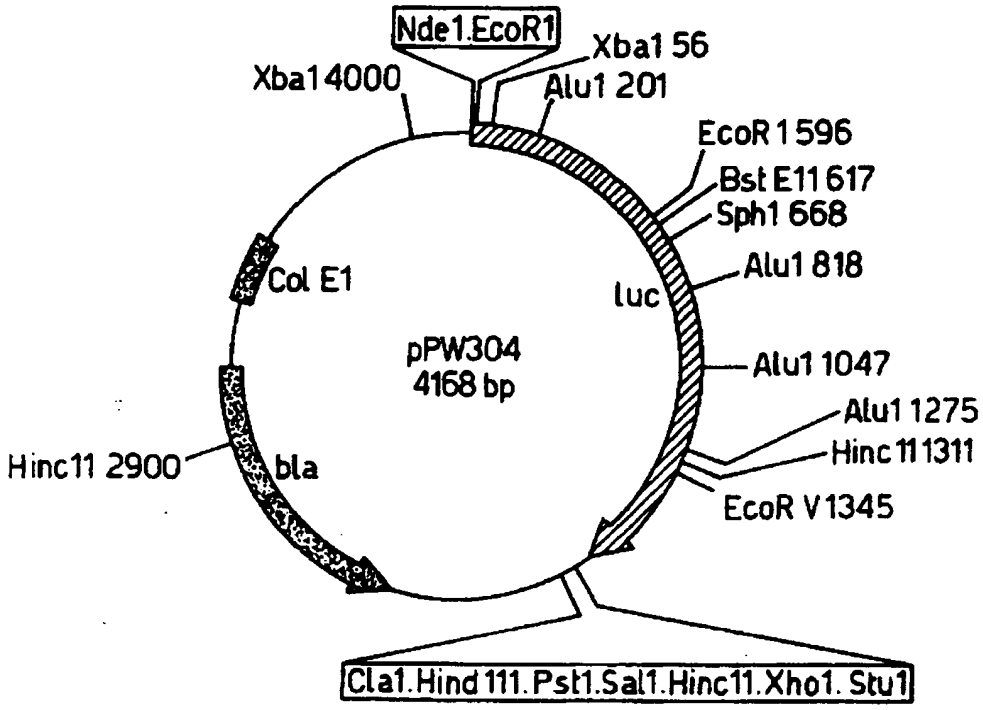
Plasmid pPW204

Fig.2.



Plasmid pPW116

Fig.3.



Plasmid pPW304

Fig.4.

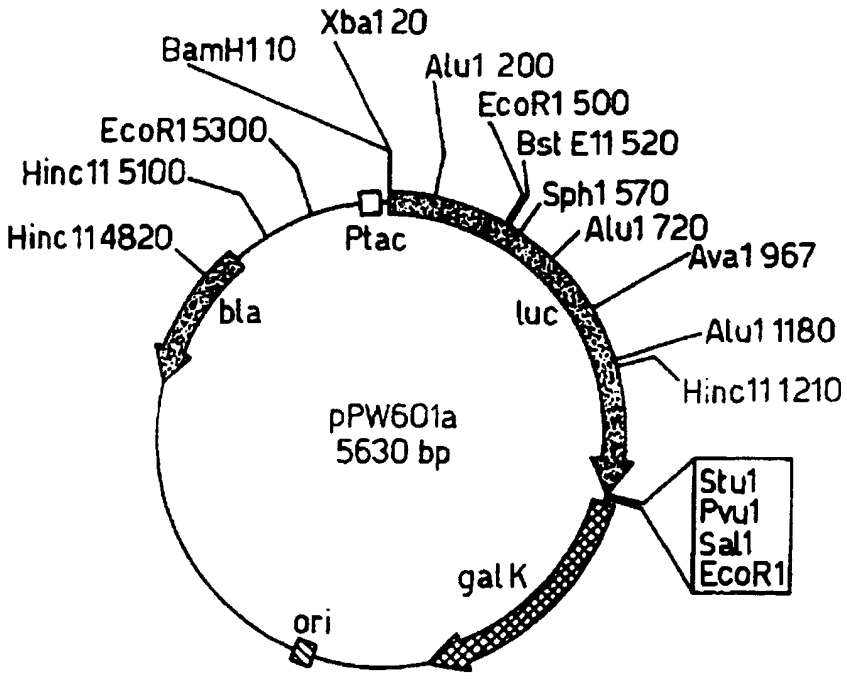


Fig.5.

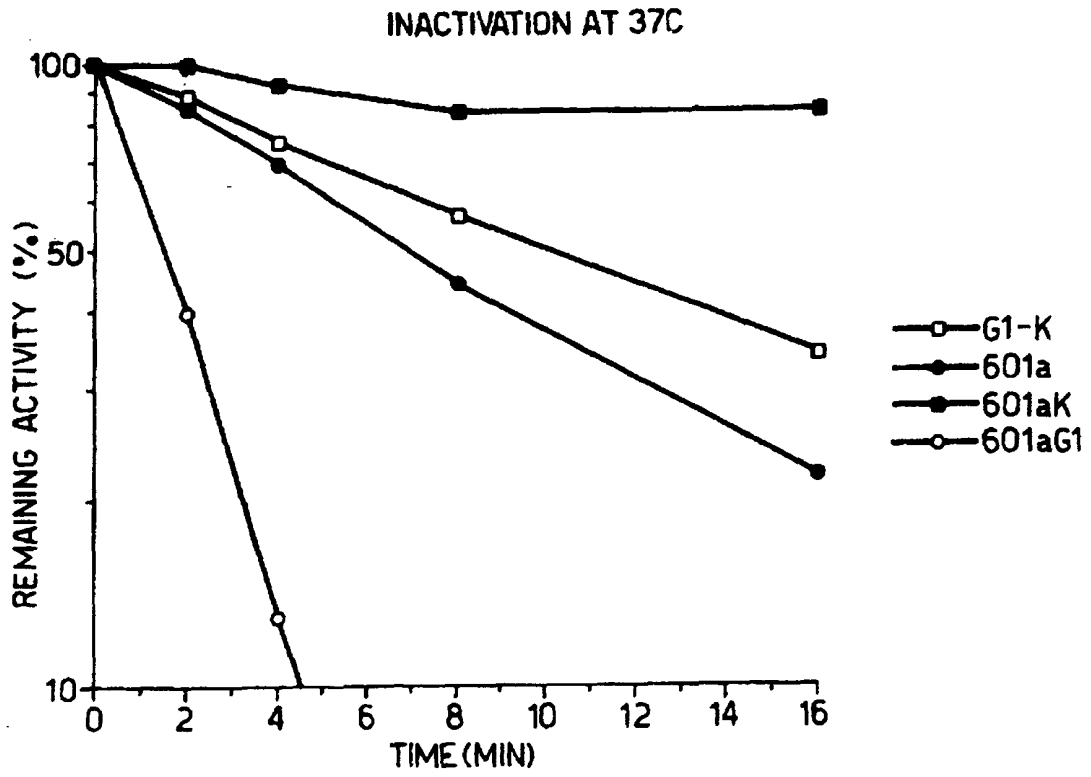

INACTIVATION AT 37C
100
50
10
REMAINING ACTIVITY (%)
0
2
4
6
8
10
12
14
16
TIME (MIN)
G1-K
601a
601aK
601aG1

Fig.6.

pT7-7

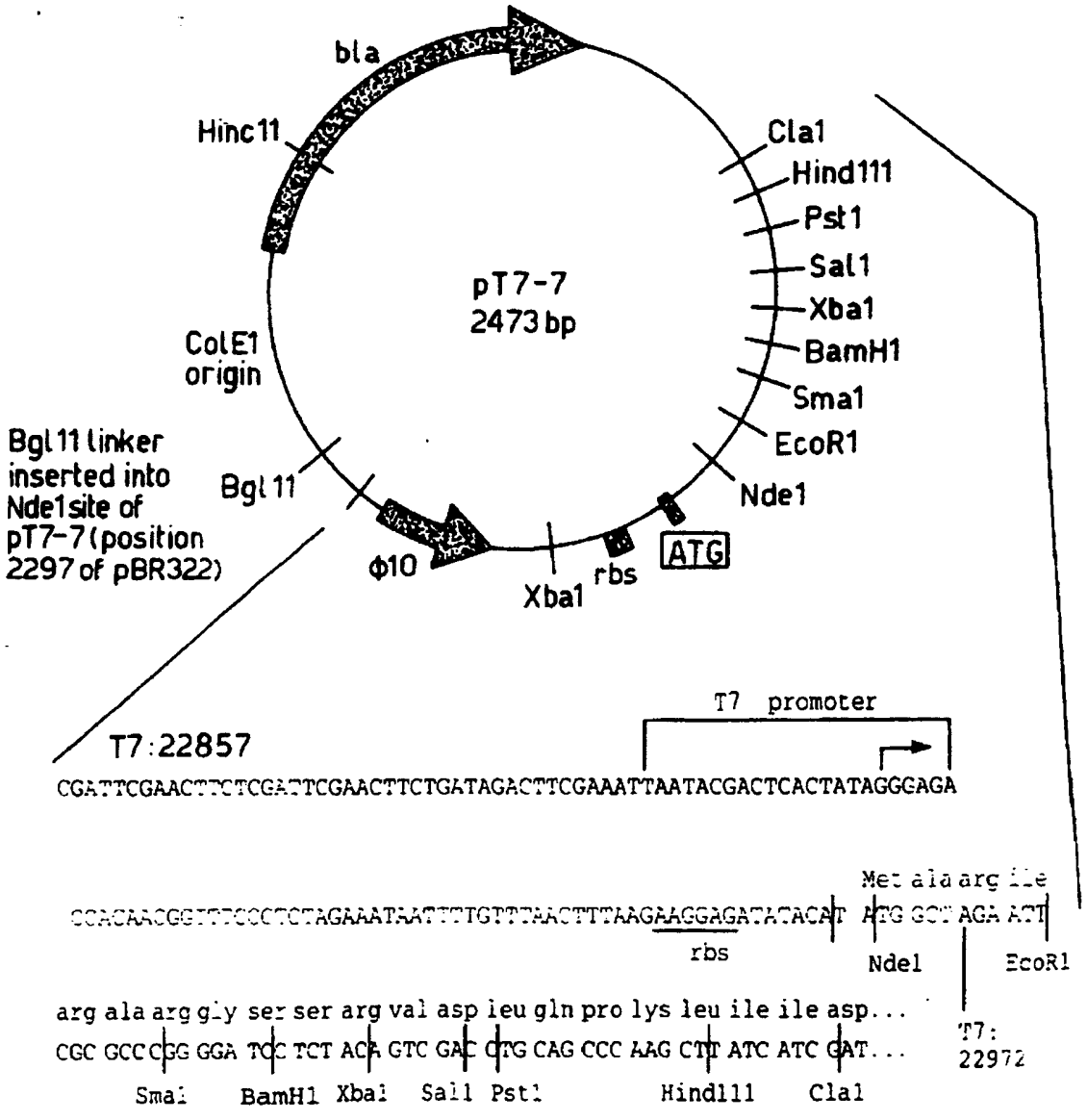
Contains T7 RNA polymerase promoter ϕ10 and the translation start site for the T7 gene 10 protein (T7 bp 22857 to 22972), inserted between the Pvu11 and the Cla1 sites of pT7-5. Unique restriction sites for creation of fusion proteins (after filling in 5' ends) are:

Frame 0: EcoR1
Frame 1: Nde1, Sma1, Cla1
Frame 2: BamH1, Sal1, Hind111

Sac1 site of original polylinker removed by deletion.
Note the additional Xba1 site upstream of start codon.

Stan Tabor
Dept. of Biol. Chem.
Harvard Medical School
Boston, Mass. 02115

November 1987



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/00099

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 C12Q1/26 C12N1/21 C12N1/19
//(C12N1/21,C12R1:19),(C12N1/19,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 524 448 (KIKKOMAN CORP) 27 January 1993 see claims	1
A	EP,A,0 449 621 (KIKKOMAN CORP) 2 October 1991 see claims	1
P,A	WO,A,95 25798 (UNITED KINGDOM SECRETARY OF STATE FOR DEFENCE, LONDON, UK) 28 September 1995 see claims 1-25	1-24
P,A	WO,A,95 18853 (PROMEGA CORP) 13 July 1995 see claims	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1996	09.05.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Delanghe, L

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter... nal Application No
PCT/GB 96/00099

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0524448	27-01-93	US-A-	5229285	20-07-93
		JP-A-	5244942	24-09-93
EP-A-0449621	02-10-91	JP-A-	3285683	16-12-91
		US-A-	5330906	19-07-94
		US-A-	5219737	15-06-93
WO-A-9525798	28-09-95	AU-B-	1954595	09-10-95
WO-A-9518853	13-07-95	AU-B-	1830395	01-08-95
		CA-A-	2157476	13-07-95
		EP-A-	0689587	03-01-96